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(54) **PEPTIDE FRAGMENTS FOR INDUCING
SYNTHESIS OF EXTRACELLULAR MATRIX
PROTEINS**

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(57) **ABSTRACT**

Short biologically active tetrapeptides are disclosed that are
comprised of the sequences GxxG and PxxP where G (gly-
cine) and P (proline) are maintained and x is a variable amino
acid. The peptides can be used singly or in combination to
stimulate production of extracellular matrix proteins in skin.
A rapid, low-cost method of producing heterogenous formu-
lations of tetrapeptides is disclosed.

13 Claims, 3 Drawing Sheets

MGPRLSVWLL LLPAALLLHE EHSRAAAKGG CAGSGCGKCD CHGVKQKQGE
RGLPLQGV I GFGM QGPEG POGPPGQKGD TGEPLPGTK GTRGPPGASG
YPGNPLPGI PGDQPPGPP GIPGCGTKG ERGPLGPPGL PGFAGNPPGP
GLPGMKGDPG EILGHVPGLM LKGERGPGI PGTGPPGLP GLOGPVGPPG
FTGPPGPPG PGPGEKQGM GLSFQCGKGD KGDQGVSGPP GVPQAQVQE
KGDFAKGEK GKGEPPGPG MPGVGEGEP GKPGPRGKPG KGDGKGEKS
PGFPGEPGYP GLIGRQGPQG EKGEAGPPGP PGIVIGTGPL GEKGERGYPG
TPGPRGEPG KGFPLPGQP GPPGLPVPGQ AGAPGPPGER GEKDRGPPG
TSLPGPSGRD GLPGPPGSPG PPGQPGYNG IVECPGPPG DQGGPIPGQ
PGFGEIGEK GKGESCLIC DIDGYRGPPG POGPPGEIGF PGQPGAKGDR
GLPGRDGVAG VEGPGTPL IGPCKAKGE GEFFYDLRLK GDKDPPGPPG
QPGMPGRAGS PGRDGHPLP GPKGSPGSGV LKGERGPPGG VGPPGSRGDT
GPPGPPGYP AGPIGDKQA GPPGPPGSPG LPGEKGEPPG IVPPLGPPGA
EGLPGSPGPP GPGDGRGPPG TPGRPGLPE KGAVGQPCIG FPGPPGPKGV
DGLPGDMGPP GTPGRPGFNG LPPNPGVQGG KGEPPGVPLP LKGLPLPGI
PGTPGEGSI GVPVPGPHG AIGPPGLQGI RGEPPGPPGLP GSVGSPGVPG
IGPPGARGPP GQGGPPGLSG PPGIKGEKE PGPGLDMPG PKGDKGAQGL
PGITGQSGLP GLPGQGGAPG IPGPPGSKGE MGVMGTPGP GSPGVPAGP
LPGEGKHGPG PGSSGPRGDP GLKGDGKDVG LPPKPGSMDK VDMGSMKQK
GDQGEKQIG PIGEKSRGD PGTGVPKGD GQAGQPGQP PKGDPGISGT
PGAPGLPGPK GSVGMGLPG TPGKGVPGI PGQGSPLP GDKAGKEK
QAGPPGIGP GLRGEKQDQG IAGPPGSGE KGEKSGIIP GMPGSPGLK
SPGSGVPGS PGLPGEKGD GLPLDGIP VKGEAGLPGT PGTPGAGQK
GEPGSDGIP SAGEKGEPL PGRGPPGPPG AKGDGSKGE VGPPGLAGSP
GPPGSKGEG FMGPPGPPG PGLPGSPGHA TEGPKGDRGP QGQPLGPLP
GPMGPPGLPG IDGVKGDKN PGWPGAPGP GPKGDPGFQG MPGIGGSPGI
TSGKGDMPG GVPFGGPK LPGLQGIKGD QGDQGVPGAK GLPGPPGPPG
PYDIKGEPP LPGPEGPPGL KGLQGLPGK GQQGVTLVG IPGPPGPPG
DGAPGQKGM GPAGPTGPRG PPGPPGPDGL PGSMGPPGTP SVDHGFLVTR
HSQTIDDPQ PSGTILYHG YSLLYVQNE RAHQDLGTA GSCLRKFTM
PFLFCNINV CNFASRNDY YWLSTPEMP MSMAPITGEN IRPFISRCV
CEAPAMVAV HSQTIQIPP PSWSSSLWIG YSEVMHTSAG AEGSGQALAS
PGSCLEFRS APFIECHGRG TCNYYANAYS FWLATIERSE MFKKPTSTL
KAGELRTHVS RCQVCMRRT

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MGPRLSVWLL LLPAALLLHE EHSRAAAKGG CAGSGCGKCD CHGVKGQKGE
RGLPGLQGV GFPGMQGPEG PQGPPGQKGD TGEPGLPGTK GTRGPPGASG
YPGNPGLPGI PGQDGPPGPP GIPGCNGTKG ERGPLGPPGL PGFAGNPGPP
GLPGMKGDPG EILGHVPGML LKGERGFPGI PGTPGPPGLP GLQGPVGPPG
FTGPPGPPGP PGPPGEKGQM GLSFQGPKGD KGDQGVSGPP GVPGQAQVQE
KGDFATKGEK GQKGEFGFQG MPGVGEKGEP GKPGPRGKPG KDGDKGEKGS
PGFPGEFGYP GLIGRQGPQG EKGEAGPPGP PGIVIGTGPL GEKGERGYPG
TPGPRGEFGP KGFPGLPQGP GPPGLPVPQG AGAPGFPGER GEKDRGFPG
TSLPGPSGRD GLPGPPGSPG PPGQPGYTNG IVECQGPPG DQGPPGIPQG
PGFIGEIGE GQKGESCLIC DIDGYRGPPG PQGPPGEIGF PGQPGAKGDR
GLPGRDGVAG VPGPQGTPL IGQPGAKGEP GEFYFDLRLK GDKGDPGFPG
QPGMPGRAGS PGRDGHPGLP GPKGSPGSVG LKGERGPPGG VGFPGRGDT
GPPGPPGYGP AGPIGDKGQA GFPGGPGSPG LPGPKEFGPK IVPLPGPPGA
EGLPGSPGFP GPQGDRGFPG TPGRPGLPGE KGAVGQPGIG FPGPPGPKGV
DGLPGDMGPP GTPGRPGFNG LPGNPGVQGG KGEFGVGLPG LKGLPGLPGI
PGTPGEKGS GVPGVPGEHG AIGPPLQGI RGEPGLP GSVGSPGVPG
IGPPGARGPP GGQGPPGLSG PPGIKGEKGF PGFPGLDMPG PKGDKGAQGL
PGITGQSGLP GLPGQQGAPG IPGFPGSKGE MGVMGTPQGP GSPGPVGAPG
LPGEKGDHGF PGSSGPRGDP GLKGDKGDVG LPGKPGSMDK VDMGSMKGQK
GDQGEKGQIG PIGEKGSRGD PGTPGVPKGD GQAGQPGQPG PKGDPGISGT
PGAPGLPGPK GSVGGMGLPG TPGEKGVPGI PGPQGSPLP GDKGAKGEKG
QAGPPGIGIP GLRGEKGDQG IAGFPGSPGE KGEKGSIGIP GMPGSPGLKG
SPGSVGYPGS PGLPGEKGD GLPGLDGIPG VKGEAGLPGT PGPTGPAGQK
GEPSDGIPG SAGEKGEPL PGRGFPGFPG AKGDKGSKGE VGFPGLAGSP
GIPGSKGEQG FMGPPGPQGQ PGLPGSPGHA TEGPKGDRGP QGQPLPGLP
GPMGPPGLPG IDGVKGDKN PGWPGAPGVP GPKGDPGFQG MPGIGGSPGI
TGSKGDMGPP GVPGFQGPKG LPGLQGIKGD QGDQGVPGAK GLPGPPGPPG
PYDIIKGEFG LPGPEGPPGL KGLQGLPGPK GQQGVTGLVG IPGPPGIPGF
DGAPGQKGEM GPAGPTGPRG FPGPPGPDGL PGSMGPPGTP SVDHGFLVTR
HSQTIDDPQC PSGTKILYHG YSLLYVQNE RAHQDLGTA GSCLRKFSTM
PFLFCNINNV CNFASRNDYS YWLSTPEPMP MSMAPITGEN IRPFISRCV
CEAPAMVMAV HSQTIQIPPC PSGWSSLWIG YSFVMHTSAG AEGSGQALAS
PGSCLEEFRS APFIECHGRG TCNYANAYS FWLATIERSE MFKKPTPSTL
KAGELRTHVS RCQVCMRRT

FIG. 1

MMSFVQKGSW	LLLALLHPTI	ILAQQEAVEG	GCSHLGQSYA	DRDVWKPEPC
QICVCDSGSV	LCDDIICDDQ	ELDCPNPEIP	FGECCAVCPQ	PPTAPTRPPN
GQGPQGPKGD	PGPPGIPGRN	GDPGIPGQPG	SPGSPGPPGI	CESCPTGPQN
YSPQYDSYDV	KSGVAVGGLA	GYPGPAGPPG	PPGPPGTSGH	PGSPGSPGYQ
GPPGEPGQAG	PSGPPGPPGA	IGPSGPAGKD	GESGRPGRPG	ERGLPGPPGI
KGPAGIPGFP	GMKGHRGFDG	RNGEKGETGA	PGLKGENGLP	GENGAPGPMG
PRGAPGERGR	PGLPGAAGAR	GNDGARGSDG	QPGPPGPPGT	AGFPGSPGAK
GEVGPAGSPG	SNGAPGQRGE	PGPQGHAGAQ	GPPGPPGING	SPGGKGEMGP
AGIPGAPGLM	GARGPPGPAG	ANGAPGLRGG	AGEPGKNGAK	GE <u>PGPRGERG</u>
EAGIPGVPGA	KGEDGKDGSF	GEPGANGLPG	AAGERGAPGF	RGPAGPNGIP
GEKGPAGERG	APGPAGPRGA	AGEPGRDGVF	GGPGMRGMPG	SPGGPGSDGK
PGPPGSQGES	GRPGPPGPSG	PRGQPGVMGF	PGPKGNDGAP	GKNGERGGPG
GPGPQGPPGK	NGETGPQGPP	GPTGPGGDKG	DTGPPGPQGL	QGLPGTGGPP
GENGKPGEPG	PKGDAGAPGA	PGGKGDAGAP	GERGPPGLAG	APGLR <u>GAGP</u>
PGPEGGKGAA	GPPGPPGAAG	TPGLQGMPGE	RGGLGSPGPK	GDKGEPGGPG
ADGVPGKDGP	RGPTGPIGPP	GPAGQPGDKG	EGGAPGLPGI	AGPRGSPGER
GETGPPGPAG	FPGAPGQNGE	PGGKGERGAP	GEKGEGGPPG	VAGPPGGSGP
AGPPGPQGVK	GERGSPGGPG	AAGFPGARGL	PGPPGSNGNP	GPPGPSGSPG
KDGPPGPAGN	TGAPGSPGVS	GPKG DAGQPG	EKGSPGAQGP	PGAPGPLGIA
GITGARGLAG	PPGM <u>PGPRGS</u>	PGPQGVKGES	GKPGANGLSG	ERGPPGPQGL
PGLAGTAGEP	GRDGNPGSDG	LPGRDGSPGG	KGDRGENGSP	GAPGAPGHFG
PPGPVGPAGK	SGDRGESGPA	GPAGAPGPAG	SRGAPGPQGP	RGDKGETGER
GAAGIKGHRG	FPGNPGAPGS	PGPAGQQGAI	GSPGPAGPRG	PVGPSGPPGK
DGTSGHFGPI	GP <u>PGPRGNRG</u>	ERGSEGSFGH	PGQPGPPGPP	GAPGPCCGGV
GAAAIAGIGG	EKAGGFAPYY	GDEPMDFKIN	TDEIMTSLKS	VNGQIESLIS
PDGSRKNPAR	NCRDLKFCHP	ELKSGEYWVD	PNQGCKLDAI	KVFCNMETGE
TCISANPLNV	PRKHWWTDSS	AEKKHVWFGE	SMDGGFQFSY	GNPELPEDVL
DVQLAFLRLL	SSRASQNITY	HCKNSIAYMD	QASGNVKKAL	KLMGSNEGEF
KAEGNSKFTY	TVLEDGCTKH	TGEWSKTVFE	YRTRKAVRLP	IVDIAPYDIG
GPDQEFQVDV	GPVCFL			

FIG. 2

MGPRLSVWLL	LLPAALLLHE	EHSRAAAKGG	CAGSGCGKCD	CHGVKGQKGE
RGLPGLQGVI	GFPGMQGPEG	PQGPPGQKGD	TGEPGLPGTK	GTRGPPGASG
YPGNPGLPGI	PGQDGP <u>PGPP</u>	GIPGCNGTKG	ERGPLGPPGL	PGFAGN <u>PGPP</u>
GLPGMKGDPG	EILGHVPGML	LKGERGFPGI	PGT <u>PGPP</u> GLP	GLQGPVGPFG
FTGPP <u>PGPP</u> GP	<u>PGPP</u> GEKGQM	GLSFQGPCKD	KGDQGVSGPP	GVPGQAQVQE
KGDFATKGEK	GQKGEFPGFQG	MPGVGEKGEF	GKPGPRGKPG	KDGDKEKGS
PGFPGEFPGY	GLIGRQGPQG	EKGEAGP <u>PGP</u>	<u>PG</u> GIVIGTGPL	GEKGERGYPG
TPGPRGEPGP	KGFPGLPGQP	GPPGLPVPQG	AGAPGFPGER	GEKGDRGFPG
TSLPGPSGRD	GL <u>PGPP</u> GSFG	PPGQPGYTNG	IVECQ <u>PGPP</u> G	DQGPPGIPGQ
PGFIGEIGEK	GQKGESCLIC	DIDGYRGPPG	PQGPPGEIGF	PGQPGAQKDR
GLPGRDGVAG	VPGPQGTPLG	IGQPGAQKGE	GEFYFDLRLK	GDKGDPGFPG
QPGMPGRAGS	PGRDGHPLGP	GPKGSPGSGV	LKGERGPPGG	VGFPGRSGDT
GP <u>PGPP</u> GYGP	AGPIGDKGQA	GFPGGPGSPG	LPGPKEGPGK	IVPL <u>PGPP</u> GA
EGLPGSPGFP	GPQGDRGFPG	TPGRPGLPGE	KGAVGQPGIG	<u>FPGPP</u> GPKGV
DGLPGDMGPP	GTPGRPFGNG	LPGNPGVQGG	KGEPGVGLPG	LKGLPGLPGI
PGTPGEKGS	GVPGVPGEHG	AIGPPGLQGI	RGE <u>PGPP</u> GLP	GSVGSPGVPG
IGPPGARGPP	GGQGPPGLSG	PPGIKGEKGF	PGFPGLDMPG	PKGDKGAQGL
PGITGQSGLP	GLPGQQGAPG	IPGFPGSKGE	MGVMGTPGQP	GSPGPVGAPG
LPGEKGDHGF	PGSSGPRGDP	GLKGDKGDVG	LPKGKPSMDK	VDMGSMKGQK
GDQGEKQIG	PIGEKGSRGD	PGTPGVPGKD	GQAGQPGQPG	PKGDPGISGT
PGAPGLPGPK	GSVGGMGLPG	TPGEKGVPGI	PGPQGSPLGP	GDKGAKGEKG
QAGPPGIGIP	GLRGEKGDQG	IAGFPGPSPE	KGEKGSIGIP	GMPGSPGLKG
SPGSVGYPGS	PGLPGEKGDK	GLPGLDGI PG	VKGEAGLPGT	PGPTGPAGQK
GEPGSDGIPG	SAGEKGEPLG	PGRGFPGFPG	AKGDKGSKGE	VGFPGLAGSP
GIPGSKGEQG	FMGPPGPQGG	PGLPGSPGHA	TEGPKGDRGP	QGQPGLPLGP
GPMGPPGLPG	IDGVKGDKN	PGWPGAPGVP	GPKGDPGFQG	MPGIGGSPGI
TGSKGDMGPP	GVPGFQGPKG	LPGLQGIKGD	QGDQGVPGAK	GL <u>PGPP</u> GPPG
PYDIIKGEFG	LPGPEGPPGL	KGLQGLPGPK	GQQGVVTGLVG	I <u>PGPP</u> GIPGF
DGAPGQKGEM	GPAGPTGPRG	<u>FPGPP</u> GPDGL	PGSMGPPGTP	SVDHGFLVTR
HSQTIDDPQC	PSGTKILYHG	YSLLYVQGNE	RAHGQDLGTA	GSCLRKFSTM
PFLFCNINNV	CNFASRNDYS	YWLSTPEPMP	MSMAPITGEN	IRPFISRCV
CEAPAMVMV	HSQTIQIPPC	PSGWSSLWIG	YSFVMHTSAG	AEGSGQALAS
PGSCLEEFRS	APFIECHGRG	TCNYYANAYS	FWLATIERSE	MFKKPTPSTL
KAGELRTHVS	RCQVCMRRT			

FIG. 3

PEPTIDE FRAGMENTS FOR INDUCING SYNTHESIS OF EXTRACELLULAR MATRIX PROTEINS

This application is a continuation of application Ser. No. 13/339,606 filed Dec. 29, 2011, now U.S. Pat. No. 8,658,764 which is a continuation of Ser. No. 11/811,876 filed Jun. 12, 2007, now U.S. Pat. No. 8,110,658 which claims benefit of priority to U.S. 60/813,284 filed Jun. 13, 2006, each of which is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

The invention relates to tetrapeptides with the amino acid motif GxxG or PxxP, where G (glycine) and P (proline) are maintained and x is a variable amino acid. The invention also relates to frame shift active tetrapeptides which are tetrapeptide sequences shifted one frame from a GxxG or PxxP tetrapeptide in an ECM protein. In particular, the invention relates to GxxG, PxxP, or frame shift active peptides that stimulate production of extracellular matrix proteins and enhance wound closure of the epithelial cell monolayer of scratch-wounded human skin. The peptide compositions may be used in formulations for repairing damaged skin or maintaining healthy skin.

BACKGROUND OF THE INVENTION

Skin aging is commonly viewed as wrinkle formation and impaired wound healing. A wound is defined as a break in the epithelial integrity of the skin. Normal wound healing involves a complex and dynamic but superbly orchestrated series of events leading to the repair of injured tissues. The largest component of normal skin is the extracellular matrix (ECM), a gel-like matrix produced by the cells that it surrounds. The ECM is composed of two major classes including fibrous structural proteins and proteoglycans. Changes in the composition and crosslinked state of the ECM are known to be associated with aging and a range of acquired and heritable skin disorders. It has been well documented that ECM not only provides structural support, but also influences cellular behavior such as differentiation and proliferation. Also, more and more research suggests that the matrix components may be a source of cell signals to facilitate epithelial cell proliferation and migration and thus enhance wound healing.

The largest class of fibrous ECM molecules is the collagen family, which includes at least 16 different types of collagen. Collagen in the dermal matrix is composed primarily of type I (80-85%) and type III (8-11%) collagens, both of which are fibrillar, or rod-shaped, collagens. The tensile strength of skin is due predominately to these fibrillar collagen molecules, which self-assemble into microfibrils in a head-to-tail and staggered side-to-side lateral arrangement. Collagen molecules become cross-linked to adjacent collagen molecules, creating additional strength and stability in collagen fibers. Damage to the collagen network (e.g. by enzymes or physical destruction), or its total collapse causes healing to take place by repair.

Various bioactive peptides that stimulate production of ECM proteins have been reported in both the scientific literature and in issued patents. Peptides historically have been isolated from natural sources and have recently been the subject of structure-function relationship studies. Natural peptides have also served as starting points for the design of synthetic peptide analogs.

Specific sequences within ECM proteins can stimulate useful elements in skin, such as type I collagen, type III collagen,

and fibronectin (Katayama et. al., J. BIOL. CHEM. 288:9941-9944 (1983)). Katayama et al. identified the pentapeptide, KTTKS (SEQ ID NO:17), within the carboxy-terminal propeptide (residues 197-241) of type I collagen. The propeptide is cleaved during production of the mature collagen protein. The cleaved propeptide may participate in regulating collagen production via a biosynthesis feedback mechanism, with the KTTKS segment playing an active role. Maquart et al. (J SOC BIOL. 193:423-28 (1999)) reported that the peptides GHK and CNYYSNS also stimulate ECM synthesis. These sequences may be released during ECM turnover, thereby signaling the need for ECM repair. The short peptide sequences liberated by either mechanism are often called "matrikines" (Maquart et al., J. SOC. BIOL. 193:423-28 (1999)).

While a number of natural and synthetic peptides exist, there is a need for improved biologically active peptides and methods for their use.

SUMMARY OF THE INVENTION

Tetrapeptides are disclosed that are characterized by the amino acid sequence motif GxxG or PxxP, where G (glycine) and P (proline) residues are maintained and x is a variable amino acid. The tetrapeptides are derived from sequences that occur multiple times throughout the primary sequence of the ECM protein, type IV collagen. The disclosed sequences induce production of all forms of collagen more than previously known peptide sequences, including KTTKS, sold under the trademark MATRIXYL™ by SEDERMA SAS (France). Further, a composition comprising a combination of various multiply-repeating sequences elicits an even greater collagen-producing response. Additional benefits may be expected from peptide combinations present in a variety of ECM proteins.

Producing a specific combination of tetrapeptides for ECM rebuilding can be commercially cost-prohibitive. A relatively simple and cost-effective means of producing a diverse combination of biologically active tetrapeptides is disclosed. By producing a combinatorial library of tetrapeptides with the GxxG or PxxP motif, a variety of biologically active tetrapeptides can be generated in the same manufacturing run (e.g., GEPG, GPEG, GPPG, and GEEG). The combination of tetrapeptides may induce more formation of ECM proteins than single peptides. Compositions comprising the disclosed tetrapeptides, alone or in combination, are useful in skin care markets including, but not limited to, those that address skin wrinkling, toning, firmness, or sagging. The stimulation of collagen by the disclosed tetrapeptides can significantly improve the health and appearance of damaged and aged skin.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 is SEQ ID NO:45 which is the Collagen IV amino acid sequence illustrating the occurrences of GxxG tetrapeptides. All bold sequences are underlined and overlapping sequences are double-underlined.

FIG. 2 is SEQ ID NO:46 which is the Collagen III amino acid sequence illustrating the occurrences of the frame shift actives PGPR and GAGP. All frame shift active sequences are bold and underlined and the GxxG sequences occurring one frame shift away are double-underlined.

FIG. 3 is also SEQ ID NO:45, the Collagen IV amino acid sequence, illustrating the occurrences of the tetrapeptide PGPP.

DETAILED DESCRIPTION OF THE INVENTION

The invention is generally directed towards tetrapeptides that stimulate production of ECM proteins and modulate wound healing, and uses of such tetrapeptides.

Peptides

One embodiment of the invention is directed towards an isolated tetrapeptide comprising the motif GxxG or PxxP. In this embodiment G (glycine) or P (proline) is maintained and x is a variable amino acid. The peptide can generally be any peptide that falls within the above description, and more preferably is SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, or SEQ ID NO:16.

Another embodiment of the invention is directed towards an isolated tetrapeptide comprising the motif GxPG, where x is P at either variable position, or both. In this embodiment, G (glycine) and P (proline) are maintained and x is a variable amino acid. The peptide can generally be any peptide that falls within the above description, and more preferably is SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5, or SEQ ID NO:7.

Another embodiment of the invention is directed towards an isolated tetrapeptide comprising the motif GExG. In this embodiment, G (glycine) and E (glutamic acid) are maintained and x is a variable amino acid. The peptide can generally be any peptide that falls within the above description, and more preferably is SEQ ID NO:5 or SEQ ID NO:8.

Another embodiment of the invention is directed towards an isolated tetrapeptide comprising the motif PGxP. In this embodiment, P (proline) and G (glycine) are maintained and x is a variable amino acid. The peptide can generally be any peptide that falls within the above description, and more preferably is SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:14, or SEQ ID NO:16.

Another embodiment of the invention is directed towards an isolated tetrapeptide comprising the motif PExP. In this embodiment, P (proline) and E (glutamic acid) are maintained and x is a variable amino acid. The peptide can generally be any peptide that falls within the above description, and more preferably is SEQ ID NO:1 or SEQ ID NO:9.

Another embodiment of the invention is directed towards a frame shift active tetrapeptide. In this embodiment, the tetrapeptide occurs one frame shift from either a GxxG or PxxP tetrapeptide in an ECM protein. The peptide can generally be any peptide that falls within the above description, and more preferably is SEQ ID NO:4 or SEQ ID NO:6.

Each of the above-described peptides can comprise D- or L-amino acids. The peptides can comprise all D-amino acids or L-amino acids. The peptides can have an acid C-terminus ($-\text{CO}_2\text{H}$) or, preferably, an amide C-terminus ($-\text{CONH}_2$, $-\text{CONHR}$, or $-\text{CONR}_2$). The peptides may be further augmented or modified, either chemically or enzymatically. For example, the peptides may be amidated ($-\text{NH}_2$) on the C-terminus, which may render the tetrapeptide less susceptible to protease degradation and increase their solubility compared to the free acid forms. The peptides may also be lipidated which may provide for enhanced skin penetration.

The above-described peptides may contain the following amino acids: R (arginine), L (leucine), P (proline), F (phenylalanine), Q (glutamine), E (glutamic acid), I (isoleucine), K (lysine), S (serine), V (valine), A (alanine), N (asparagine), D (aspartic acid), T (threonine), Y (tyrosine) and G (glycine). The above-described peptides do not include the following M (methionine), C (cysteine), H (histidine) or W (tryptophan).

Accordingly, in one embodiment, x is not selected from either (methionine), C (cysteine), H (histidine) or W (tryptophan). Methods of Use

An additional embodiment of the invention is directed towards methods of using the above-described peptides. The methods of use may involve the use of a single peptide, or may involve the use of two or more peptides in combination.

An embodiment of the invention is a method of promoting repair of damaged skin and maintenance of healthy skin using tetrapeptides that stimulate production of ECM proteins. The method generally is directed towards contacting dermal (skin) cells with a composition containing the peptide. The compositions can be an aerosol, emulsion, liquid, lotion, cream, paste, ointment, foam, or other pharmaceutically acceptable formulation. Generally, a pharmaceutically acceptable formulation would include any acceptable carrier suitable for use on human skin, e.g. cosmetically acceptable carrier and dermatological acceptable carrier. The compositions may contain other biologically active agents such as retinoids or other peptides. The compositions may contain pharmaceutically acceptable carriers or adjuvants. The contacting step can be performed in vivo, in situ, in vitro, or by any method known to those of skill in the art. Most preferably, the contacting step is to be performed topically at a concentration sufficient to elicit a stimulatory response. The concentration of the peptide in the composition can be about 0.01 $\mu\text{g/mL}$ to about 100 $\mu\text{g/mL}$, about 0.1 $\mu\text{g/mL}$ to about 50 $\mu\text{g/mL}$, and about 0.1 $\mu\text{g/mL}$ to about 1 $\mu\text{g/mL}$. The contacting step can be performed on a mammal, a cat, a dog, a cow, a horse, a pig, or a human. A preferred composition for promoting ECM protein production comprises SEQ ID NO:8; more preferably, the composition comprises SEQ ID NO:8 in a heterogeneous mixture with at least one other tetrapeptide. In a most preferred embodiment, the individual tetrapeptides in the composition would cause sustained collagen production over a period of at least 48 hours.

An additional embodiment of the invention is directed towards a method for promoting wound healing of skin damaged by normal aging, disease, injury, trauma, or by surgery or other medical procedures. The method can comprise administering to the wound of an animal a composition, wherein the composition comprises any of the above-described peptides, singularly or in combination. The compositions can be a liquid, lotion, cream, paste, ointment, foam, or any other pharmaceutically acceptable formulation. The compositions may contain pharmaceutically acceptable carriers or adjuvants. The compositions may contain other biologically active agents such as antimicrobial agents or growth factors. The compositions may also be used in combination with other therapeutic agents such as tissue grafts, tissue culture products, oxygen or dressings. The concentration of the peptide in the composition can be about 0.01 $\mu\text{g/mL}$ to about 100 $\mu\text{g/mL}$, about 0.1 $\mu\text{g/mL}$ to about 50 $\mu\text{g/mL}$, and about 0.1 $\mu\text{g/mL}$ to about 1 $\mu\text{g/mL}$. The composition can be administered to the wound topically. The animal can generally be any kind of animal, and preferably is a mammal, and more preferably is a human, cow, horse, cat, dog, pig, goat, or sheep. A preferred composition for wound healing applications in which ECM protein production is promoted comprises SEQ ID NO:8; more preferably, the composition comprises SEQ ID NO:8 in a heterogeneous mixture with at least one other tetrapeptide. In a most preferred embodiment, the individual tetrapeptides in the composition would cause sustained collagen production over a period of at least 48 hours.

An additional embodiment of the invention is directed towards a method for reducing scarring of skin damaged by normal aging, disease, injury, trauma, or by surgery or other

medical procedures. The method can comprise administering to the wound of an animal a composition, wherein the composition comprises any of the above-described peptides, singularly or in combination. The compositions can be a liquid, lotion, cream, paste, ointment, foam, or other pharmaceutically acceptable formulation. The compositions may contain pharmaceutically acceptable carriers or adjuvants. The compositions may contain other biologically active agents such as antimicrobial agents or growth factors. The compositions may also be used in combination with other therapeutic agents such as tissue grafts, tissue culture products, oxygen or dressings. The concentration of the peptide in the composition can be about 0.01 µg/mL to about 100 µg/mL, about 0.1 µg/mL to about 50 µg/mL, and about 0.1 µg/mL to about 1 µg/mL. The composition can be administered to the wound topically. The animal can generally be any kind of animal, and preferably is a mammal, and more preferably is a human, cow, horse, cat, dog, pig, goat, or sheep. A preferred composition for wound healing applications in which ECM protein production is promoted comprises SEQ ID NO:8; more preferably, the composition comprises SEQ ID NO:8 in a heterogeneous mixture with at least one other tetrapeptide. In a most preferred embodiment, the individual tetrapeptides in the composition would cause sustained collagen production over a period of at least 48 hours.

A further embodiment of the invention is directed towards a method for producing the disclosed tetrapeptides in combination. The peptides may be produced using any method known to those skilled in the art such as those disclosed in Merrifield, R. B., *Solid Phase Peptide Synthesis I*, J. AM. CHEM. SOC. 85:2149-2154 (1963); Carpino, L. A. et al., [(9-Fluorenylmethyl)Oxy]Carbonyl (Fmoc) Amino Acid Chlorides: Synthesis, Characterization, And Application To The Rapid Synthesis Of Short Peptides, J. ORG. CHEM. 37:51:3732-3734; Merrifield, R. B. et al., *Instrument For Automated Synthesis Of Peptides*, ANAL. CHEM. 38:1905-1914 (1966); or Kent, S. B. H. et al., *High Yield Chemical Synthesis Of Biologically Active Peptides On An Automated Peptide Synthesizer Of Novel Design*, IN: PEPTIDES 1984 (Ragnarsson U., ed.) Almqvist and Wiksell Int., Stockholm (Sweden), pp. 185-188, all of which are incorporated by reference herein in their entirety. Preferably, the peptides will be produced by a machine capable of sequential addition of amino acids to a growing peptide chain. However, the peptides may also be manufactured using standard solution phase methodology.

It has been observed that the addition of a mixture of free amino acids instead of homogenous peptide mixtures during peptide chain synthesis results in varied incorporation of free amino acids such that a combination of peptides results from the synthesis reactions. The relative incorporation frequency of a particular amino acid included in a mixture of two or more amino acids added during synthesis may be adjusted. Adjustment is made possible by modifying the ratio of a free

amino acid made available during the synthesis process relative to the other amino acids in the mixture (this is termed an isokinetic mixture).

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLES

Example 1

Identification of Repeat Tetrapeptide Sequences in Collagen

A relatively high proportion of collagen IV tetrapeptide repeat sequences have the motif GxxG (where x is any amino acid). A number of these are shown in situ as part of the full collagen IV sequence illustrated in FIG. 1 as SEQ ID NO:45. Collagen IV was examined first due to its role of interacting with other specialized ECM components (See Gregory Schultz et al., 2005). There are eleven sequences with the GxxG motif in collagen IV that appear more than ten times (GxxG where xx is represented by: vp, ek, fp, vp, pp, sp, ep, lp, pk, qp and tp). Of these tetrapeptide sequences, eight of eleven sequences contain proline in position 3, two of eleven sequences contain P in position 2, one of eleven sequences contains proline in positions 2 and 3, and one of eleven sequences contains no proline. The disclosed sequences are referred to as REPLIKINES™. "REPLIKINE" is defined as a short sequence within ECM proteins that occurs multiple times (i.e., is replicated). This sequence may be present in one ECM protein (e.g., collagen IV). Preferably, the sequence is present in multiple ECM proteins (e.g., all collagens, elastin, laminin, etc.). The presence of the sequence in multiple ECM proteins increases the likelihood that the fragment may be able to promote ECM synthesis or repair.

The eleven GxxG sequences appearing in collagen IV listed above are highlighted in the human collagen IV sequence illustrated in FIG. 1. In this figure, all bold sequences are underlined and overlapping sequences are double-underlined. All but one of these sequences also appears in collagens I, II, III, and V. This fact contributes to the ability of the disclosed peptides to stimulate the production of all collagen types, particularly when the peptides are used in combination. Table 1 shows the frequency of several tetrapeptide repeats in ECM proteins. Bold sequences in Table 1 are those that appear in collagen IV ten or more times.

TABLE 1

Frequency of tetrapeptides in ECM proteins								
SEQ. ID NO	Sequence	Collagen I	Collagen II	Collagen III	Collagen IV	Collagen V	Elastin Precursor	
19	GAAG	10	5	7		2	4	5
20	GAKG	3	4	3	5	5		
21	GAPG	13	21	25	6	9		
22	GDKG	2	2	4	9	3		

TABLE 1-continued

Frequency of tetrapeptides in ECM proteins								
SEQ. ID	Sequence	Collagen I	Collagen II	Collagen III	Collagen IV	Collagen V	Elastin	Elastin Precursor
NO								
23	GDRG	2	5	2	4	1		
8	GEKG	3	5	4	22	15		
5	GEPG	11	15	10	11	4		
24	GERG	10	11	14	6	7		
2	GFPG	4	8	6	22	5	1	1
25	GIPG	2	2	6	14	6	5	5
26	GKDG	1	4	5	2	2		
27	GKPG	2	3	3	4	1		
28	GLKG	2	1	1	5	4		
29	GLPG	15	10	9	42	15	1	1
30	GNPG	3	5	3	2	1		
31	GPAG	16	20	20	3	6		
32	GPKG	3	11	4	12	9		
7	GPPG	33	40	40	46	43		
33	GPQG	7	11	9	7	5		
34	GPRG	11	13	10	4	7		
35	GPSG	10	11	5	1	5		
36	GPTG	4	3	2	2	6		
37	GPVG	9	3	3	2	5		
38	GQPG	3	4	6	12	7		
39	GRDG	4	2	3	3			
40	GRPG	3	3	4	2	5		
3	GSPG	4	6	21	16	3		
41	GTPG	3	4	2	11	2		
42	GVKG	1	3	2	3	1		
43	GVPG		1	3	10	1	14	15
44	GYPG	1	1	1	4	2		

As also evident from a review of the collagen IV sequence, SEQ ID NO:45, there are also many occurrences of sequences having the PxxP motif. For example, the sequence PGPP occurs no less than fifteen times as illustrated in FIG. 3. Therefore, this disclosed sequence is also referred to as a REPLIKINE™. Preferably, this sequence is present in multiple ECM proteins (e.g., all collagens, elastin, laminin, etc.) as the presence of this sequence in multiple ECM proteins increases the likelihood that the fragment may be able to promote ECM synthesis or repair. The fifteen PGPP sequences appearing in collagen IV listed above are highlighted and underlined in the human collagen IV sequence illustrated in FIG. 3.

Example 2

Identification of Frame Shift Actives

In addition to the relatively high proportion of collagen IV tetrapeptide repeat sequences with the motif GxxG, other tetrapeptide sequences occurring one amino acid frame shift away from a GxxG or PxxP tetrapeptide sequence have been identified. These sequences may repeat or occur only once within an ECM protein and may be located one amino acid position away from either a GxxG or PxxP tetrapeptide sequence as described herein. These tetrapeptide sequences are referred to as frame shift actives. Such frame shift actives may accordingly contain either a G or a P in either the second or third position depending on the direction of frame shift. It

has been further recognized that frame shift actives may be combined with other tetrapeptide sequences disclosed in this application forming a combikine. An example of such a combikine is H06 and H15.

One example of a frame shift active is GAGP or H12 (SEQ ID NO:6). H12 (GAGP) appears one residue (or frame) shift from the GxxG tetrapeptide GGAG in Collagen III (SEQ ID NO:46) as illustrated in FIG. 2. In this figure, all frame shift active sequences are bold and underlined and the GxxG sequences occurring one frame shift away are double-underlined. Furthermore, as shown in Table 5, this tetrapeptide (GAGP) achieves good results for collagen production at 48 hours. Another example is the sequence PGPR, which is H10 (SEQ ID NO:4) which occurs eleven times in Collagens I-IV. As it appears multiple times in an individual ECM protein, this tetrapeptide would further be considered a REPLIKINE. FIG. 2 (SEQ ID NO:46) illustrates several instances of this tetrapeptide with each occurring one frame shift from the GxxG tetrapeptide GPRG. This particular frame shift active appears in multiple ECM proteins and therefore increases the likelihood that the fragment may be able to promote ECM synthesis or repair.

Example 3

Identification of Repeat Sequences that Stimulate Collagen Production

Several sequences identified in Examples 1 and 2 were synthesized using standard peptide chemistry and assayed for the stimulation of collagen from dermal fibroblasts. The synthesized peptides were amidated at the C-terminus, which rendered the tetrapeptides less susceptible to protease degradation and increased their solubility compared to the free acid forms. Human dermal fibroblasts were incubated in 96-well plates at 37° C. and 5% CO₂ for 24 and 48 hours in 150 µL complete cell culture media (Cascade Biologics, Portland, Oreg.; Cat. No. M-106-500), supplemented with Low Serum Growth Supplement (Cascade Biologics, Portland, Oreg.; Cat. No. S-003-10) containing sample peptides at a final peptide concentration of 50 µg/mL. Each well was seeded with 10,000 cells. Following the incubation, 100-µL medium samples were recovered from each well and assayed for collagen production

The assays were performed by Tebu-bio Laboratories (France) using the SIRCOL™ Collagen Assay Kit (Biocolor Assays, UK) following the manufacturer's protocol. The SIRCOL™ Collagen Assay is a quantitative dye-binding method designed for the analysis of soluble collagens released into culture medium by mammalian cells during in vitro culture. The collagen of the tested samples binds to the anionic SIRCOL™ dye. The collagen-dye complexes precipitate out of solution and are pelleted by centrifugation. The recovered collagen-dye pellet was dissolved in an alkaline solution prior to absorbance measurements. Duplicate measurements were taken at the 24 and 48 hour times from two separate samples. The four measurements for each sample were averaged. The absorbance of reagent blanks, collagen standards, and samples were measured at 560 nm. The reagent blank absorbance was subtracted from the absorbance from each sample at 24 and 48 hours.

Two separate data sets were used to generate two collagen standard calibration curves. The first calibration curve was generated for purposes of calculating the quantity of collagen in samples H6 (combination of SEQ ID NOs:1-4), H7-H14 (SEQ ID NOs:1-8, respectively) and H15 (combination of SEQ ID NOs:5-8). The second calibration curve was gener-

ated for calculating the quantity of collagen in samples H16 (SEQ ID NO:9), H21-23 (SEQ ID NOs:10-12, respectively), H25-26 (SEQ ID NOs:13-14, respectively), or H29-30 (SEQ ID NOs:15-16, respectively), H32 (SEQ ID NO:17), H33 (combination of SEQ ID NOs:9-12), H34 (combination of SEQ ID NOs:11-14), H35 (combination of SEQ ID NOs:13-16), H36 (combination of SEQ ID NOs:1, 6, 5, 8), H37 (SEQ ID NO:17) and H38 (SEQ ID NO:8) from the absorbance measurements was created by plotting the Abs_{560nm} of the known collagen standards versus the respective concentrations of the collagen standards (in micrograms) each time a series of assays were performed. With respect to each data set, the same calibration curve was used for samples taken at the 24 and 48 hour times (Tables 2A and 2B). Accordingly, different standard curves were prepared immediately prior to performing each series of assays.

TABLE 2A

Calibration curve for assaying collagen production by peptides H6-H15		
Collagen standards (µg)	A _{560 nm} 24 h test	A _{560 nm} 48 h test
0	0.00	0.00
5	0.08	0.10
10	0.11	0.15
25	0.32	0.35
50	0.66	0.65

TABLE 2B

Calibration curve for assaying collagen production by peptides H16, H21-23, H25-26, and H29-38		
Collagen Standards (µg)	A _{560 nm} Assay date 1	A _{560 nm} Assay date 2
0	0.00	0.00
5	0.12	0.09
10	0.14	0.15
25	0.48	0.42
50	0.88	0.80

A linear regression was performed from plotting the Abs_{560nm} values versus concentrations of the respective collagen standards using MICROSOFT EXCEL™. The regression resulted in a lines described by the formula y=0.013x for both incubation times noted in Table 2A. As the results were identical, only the 24-hour time period was used for the second series calibration curves. The formula of the line obtained on assay date 1 and assay date 2 of the second series of samples was y=0.0178x and y=0.0162x, respectively. The peptide LL-37 (SEQ ID NO:18) was used as a positive control as it has been widely reported to have an impact upon wound healing in man (Heilborn et al., The Cathelicidin Anti-Microbial Peptide LL-37 Is Involved In The Re-Epithelialization Of Human Skin Wounds And Is Lacking In Chronic Ulcer Epithelium, *J. Invest. Dermato.* 120:379-89 (2003)). The assay detection limit defined by the manufacturer is 2.5 µg.

The total amount of collagen produced in samples containing peptides was calculated from the averaged absorbance values taken at 24 hours (Table 3A) and 48 hours (Table 3B) using the linear equation derived from the standard curve. The total amount of collagen produced in samples containing peptides H16 (SEQ ID NO:9), H21-23 (SEQ ID NOs:10-12, respectively), H25-26 (SEQ ID NOs:13-14, respectively), or

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H29-30 (SEQ ID NOs:15-16, respectively), H32 (SEQ ID NO:17), H33 (combination of SEQ ID NOs:9-12), H34 (combination of SEQ ID NOs:11-14), H35 (combination of SEQ ID NOs:13-16), H36 (combination of SEQ ID NOs:1, 6, 5, 8), H37 (SEQ ID NO:17) and H38 (SEQ ID NO:8) was calculated from the absorbance values taken at 24 hours (Table 4A) and 48 hours (Table 4B) using the linear equation derived from the standard curve. These values were compared with peptide LL37 (SEQ ID NO:18), a peptide known to stimulate collagen. In each table, samples marked by an asterisk (*) may not be significant as the assay detection limit is 2.5 µg.

TABLE 3A

Absorbance measurements and quantification of collagen in test samples H6-H15 at 24 hours.						
SEQ ID NO	Peptides	A _{560 nm}	Average	Average minus blank	Collagen (µg)	
18	LL37	0.102	0.136	0.12	0.04	3.0
—	H6	0.084	0.140	0.11	0.03	2.5
1	H7	0.098	0.063	0.08	0.00	0.0*
2	H8	0.122	0.078	0.10	0.02	1.5*
3	H9	0.147	0.104	0.13	0.05	3.5
4	H10	0.103	0.146	0.12	0.04	3.4
5	H11	0.110	0.168	0.14	0.06	4.5
6	H12	0.063	0.101	0.08	0.00	0.2*
7	H13	0.114	0.093	0.10	0.02	1.8*
8	H14	0.115	0.122	0.12	0.04	3.0
—	H15	0.132	0.093	0.11	0.03	2.5
—	Blank	0.074	0.076	0.08	0.00	0.0

TABLE 3B

Absorbance measurements and quantification of collagen in test samples H6-H15 at 48 hours.						
SEQ ID NO	Peptides	A _{560 nm}	Average	Average minus blank	Collagen (µg)	
18	LL37	0.262	0.113	0.19	0.07	5.2
—	H6	0.086	0.189	0.14	0.02	1.3*
1	H7	0.192	0.189	0.19	0.07	5.4
2	H8	0.137	0.126	0.13	0.01	0.9*
3	H9	0.117	0.061	0.09	0.00	0.0*
4	H10	0.136	0.085	0.11	0.00	0.0*
5	H11	0.113	0.181	0.15	0.03	2.1*
6	H12	0.106	0.231	0.17	0.05	3.7
7	H13	0.100	0.145	0.12	0.00	0.2*
8	H14	0.132	0.176	0.15	0.03	2.6
—	H15	0.177	0.174	0.18	0.06	4.3
—	Blank	0.120	0.115	0.12	0.00	0.0

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TABLE 4A

Absorbance measurements and quantification of collagen in test samples H16, H21-23, H25-26, or H29-38 at 24 hours.						
SEQ ID NO	Peptides	A _{560 nm}	Average	Average minus blank	Collagen (µg)	
9	H16	0.133	0.137	0.14	0.06	3.1
10	H21	0.129	0.119	0.12	0.04	2.5
11	H22	0.192	0.085	0.14	0.06	3.3
12	H23	0.090	0.073	0.08	0.00	0.1*
13	H25	0.129	0.076	0.10	0.02	1.3*
14	H26	0.114	0.149	0.13	0.05	2.9
15	H29	0.111	0.063	0.09	0.01	0.4*
16	H30	0.099	0.092	0.10	0.02	0.9*
17	H32	0.087	0.055	0.07	−0.01	−0.5*
—	(crystals and cell toxicity)					
—	H33	0.086	0.125	0.11	0.03	1.4*
—	H34	0.117	0.120	0.12	0.04	2.2*
—	H35	0.103	0.090	0.10	0.02	0.9*
—	H36	0.105	0.128	0.12	0.04	2.1*
17	H37	0.099	0.100	0.10	0.02	1.1*
8	H38	0.103	0.159	0.13	0.05	2.9
—	Blank	0.072	0.086	0.08	0.00	0.0

TABLE 4B

Absorbance measurements and quantification of collagen in test samples H16, H21-23, H25-26, or H29-38 at 48 hours.						
SEQ ID NO	Peptides	A _{560 nm}	Average	Average minus blank	Collagen (µg)	
9	H16	0.065	0.064	0.06	0.00	0.3*
10	H21	0.089	0.126	0.11	0.05	2.9
11	H22	0.102	0.087	0.09	0.03	2.1*
12	H23	0.093	0.082	0.09	0.03	1.7*
13	H25	0.059	0.084	0.07	0.01	0.7*
14	H26	0.081	0.153	0.12	0.06	3.5
15	H29	0.086	0.094	0.09	0.03	1.9*
16	H30	0.083	0.101	0.09	0.03	2.0*
17	H32	0.088	0.072	0.08	0.02	1.2*
—	(crystals and cell toxicity)					
—	H33	0.096	0.092	0.09	0.03	2.1*
—	H34	0.076	0.155	0.12	0.06	3.4
—	H35	0.120	0.074	0.10	0.04	2.3*
—	H36	0.154	0.082	0.12	0.06	3.6
17	H37	0.078	0.114	0.10	0.04	2.2*
8	H38	0.123	0.089	0.11	0.05	2.8
—	Blank	0.106	0.0106	0.06	0.00	0.0

Because sample sizes were 100 µL, the concentration of collagen produced in each sample in micrograms per milliliter is determined by multiplying the amount of collagen detected by ten. The results of all samples tested are summarized in Table 5.

TABLE 5

Collagen synthesis induced by peptides					
SEQ ID				Collagen produced (µg/mL)	
				[Peptide]	
NO	Name	Primary sequence	(µg/mL)	24 hrs	48 hrs
1	H07	PEGP	50	0	54
2	H08	GFPG	50	15	9
3	H09	GSPG	50	35	0

TABLE 5-continued

Collagen synthesis induced by peptides					
SEQ ID			[Peptide]	Collagen produced	
				(μg/mL)	
NO	Name	Primary sequence	(μg/mL)	24 hrs	48 hrs
4	H10	PGPR	50	34	0
—	H06	H7, H8, H9, H10 (SEQ ID NOs: 1, 2, 3, 4)	50	25	13
5	H11	GEPG	50	45	21
6	H12	GAGP	50	2	37
7	H13	GPPG	50	18	2
8	H14	GEKG	50	30	26
8	H38	GEKG	0.3	29	28
—	H15	H11, H12, H13, H14 (SEQ ID NOs: 5, 6, 7, 8)	50	25	43
9	H16	PEKP	50	31	3
10	H21	PKGP	50	25	29
11	H22	PGQP	50	33	21
12	H23	PGTP	50	1	17
13	H25	PMGP	50	13	7
14	H26	PGPP	50	29	35
15	H29	PQGP	50	4	19
16	H30	PGNP	50	9	20
17	H32	KTTKS (SEDERMA™ peptide)	50	na	12
17	H37	KTTKS (SEDERMA™ peptide)	0.3	11	22
—	H33	H16, H21, H22, H23 (SEQ ID NOs: 9, 10, 11, 12)	50	14	21
—	H34	H22, H23, H25, H26 (SEQ ID NOs: 11, 12, 13, 14)	50	22	34
—	H35	H25, H26, H29, H30 (SEQ ID NOs: 13, 14, 15, 16)	50	9	23
—	H36	H7, H12, H11, H14 (SEQ ID NOs: 1, 6, 5, 8)	50	21	36
18	LL37	LLGDFFRKSKEKIGKEFKRIVQRIIDFLRNLVPRTES	50	30	52

All tetrapeptides tested stimulated the production of soluble collagen. Of the sequences tested, GxxG tetrapeptides with a glutamic acid in position 2 best stimulate collagen at both 24 and 48 hour time-points. These sequences are H11 (GEPG; SEQ ID NO:5), H14 (GEKG; SEQ ID NO:8) and H38 (GEKG; SEQ ID NO:8). The peptides were initially screened using a peptide concentration of 50 μg/mL. To survey the concentration effective for stimulating collagen production, H14 (SEQ ID NO:8) was also tested at 0.3 μg/mL as H38. As shown in Table 5, H38-induced collagen stimulation was not diminished at the lower concentration, indicating that the maximal stimulating concentration of SEQ ID NO:8 is at or below 0.3 μg/mL.

To test its efficacy, SEQ ID NO:8 (H14 and H38) was compared to the peptide, LL37, (SEQ ID NO:18) which is known to stimulate collagen production. Based on the amount of collagen released by fibroblasts in response to LL37, 25 μg/mL was considered a significant amount of collagen released due to contact with a tetrapeptide. SEQ ID NO:8 induced about the same amount of collagen as LL37 (SEQ ID

NO:18) at 24 hours. Importantly, collagen produced as a result of contact with SEQ ID NO:8 was substantially maintained for at least 48 hours. SEQ ID NO:8 was also compared to a leading skin care peptide known to stimulate collagen production, KTTKS (SEQ ID NO:17) (Katayama et. al., J. BIOL. CHEM. 288:9941-9944 (1983)). KTTKS is an ingredient in the product MATRIXYL™ (SEDERMA SAS, France). SEQ ID NO:8 stimulated more collagen production than the KTTKS (SEQ ID NO:17) peptide (Table 5) at 24 and 48 hours.

Example 4

Identification of Peptide Combinations that Synergistically Enhance Collagen Stimulation—COMBIKINES

Heterogeneous populations of active tetrapeptides may stimulate collagen production at a higher level than homogeneous samples of tetrapeptides. The components of the het-

erogeneous composition are called COMBIKINES™. COMBIKINES are a group of REPLICINES combined to produce a greater or broader effect upon one or more target cell types. The peptides H11 (SEQ ID NO:5), H12 (SEQ ID NO:6), H13 (SEQ ID NO:7), and H14 (SEQ ID NO:8) were combined to a final concentration of 50 µg/mL and assayed using the same protocol as for the individual peptides. As expected, the result obtained at the 24 hour time point equaled the mean of the individual induction scores. The combination of peptides at 48 hours, however, induced collagen to a level of 43 µg/mL. Surprisingly, this amount was far in excess of the anticipated mean (21 µg/mL) of the four individual peptides (see Table 5). Thus, specific combinations of peptides may stimulate collagen production to a greater degree than the individual peptides at the same concentration. Further, tetrapeptides from a variety of ECM sources such as collagen, laminin, and elastin may produce enhanced induction of a variety of ECM proteins (see Tables 1 and 5).

Example 5

Cost-Effective COMBIKINE Manufacturing for Enhancing Stimulation of Collagen Production

The high cost of peptide synthesis limits the feasibility of producing of heterogeneous compositions of bioactive peptides. The present invention greatly mitigates this limitation. Because the presently disclosed sequences have a commonality (e.g., a glycine or proline at both termini), a range of tetrapeptides varied at positions 2 and 3 can be synthesized in a single manufacturing run. The synthetic peptides can be made by any method known in the art. (Benoiton, N., *Chemistry of Peptide Synthesis*, CRC (2005)). During manufacture

of the peptides, amino acid mixtures are added instead of homogenous samples. The chemistry for determining the correct ratios of amino acid concentrations added at the mixed positions to gain the desired ratio of resulting peptides has been described previously (Greenbaum et al., *Molecular and Cellular Proteomics* 1:60-68, 2002; Krstenansky et al., *Letters in Drug Design and Discovery* 1:6-13, 2004; both of which references are incorporated herein in their entirety). Using this methodology, a library of heterogeneous peptides can be made for nearly the same cost of synthesizing one peptide.

The application of this manufacturing process enables the cost-effective production of bioactive combikines. This is made possible by the unique composition of the disclosed tetrapeptides. The tetrapeptide mixtures are better suited for incorporation into topical use formulations than longer peptides. Because of their length, tetrapeptides have practical and chemical advantages over longer peptides, including the following: easier incorporation and dissolution into formulations, higher skin and pore permeability, and higher production yields with easier methods of manufacturing combinations of peptides. Although not required, the ideal formulations of tetrapeptides, singly or in combination, are formulations that maintain significant collagen production at 24 hours for up to 48 hours. More preferably, the formulations would induce synthesis of ECM for the entire 48 hour period such that more collagen is produced by 48 hours than at 24 hours. Although within the scope of the current invention, tetrapeptides that promote production of ECM proteins at 24 hours, but show diminished production at 48 hours, are less favored. In this regard, Table 6 shows the results of the currently disclosed peptides. Preferred peptides are in bold.

TABLE 6

Disclosed peptides					
SEQ ID NO	Peptides	Released collagen (µg/mL) 24 h	Released collagen (µg/mL) 48 h	Significant release of collagen at 24 h and 48 h	Increase in collagen release at 48 h v. 24 h
18	LL37	30	52	✓	✓
—	H6	25	13		
1	H7	0	54		✓
2	H8	15	9		
3	H9	35	0		
4	H10	34	0		
5	H11	45	21		
6	H12	2	37		✓
7	H13	18	2		
8	H14	30	26	✓	
8	H38	29	28	✓	
—	H15	25	43	✓	✓
9	H16	31	3		
10	H21	25	29	✓	
11	H22	33	21		
12	H23	1	17		✓
13	H25	13	7		
14	H26	29	35	✓	
15	H29	4	19		✓
16	H30	9	20		✓
17	H32	NA	12		
	(crystals and cell toxicity)				
17	H37	11	22		✓
—	H33	14	21		✓
—	H34	22	34		✓
—	H35	9	23		✓
—	H36	21	36		✓

Collagen Stimulators Also Serve as Multi-Effector Molecules Enhancing Skin Epithelial Cell Wound Closer

Collagens are key components of all phases of wound healing. Stimulation of collagen production reflects that damage has occurred to the collagen network (e.g. by enzymes or physical destruction). Indeed, the total collapse of the collagen network in fact causes healing to take place. Therefore a collagen stimulator may also serve as a multi-effector molecule orchestrating certain matrix remodeling and enhancing wound healing.

Wound healing experiments were performed on monolayers of human skin epithelial cells (CRL-2592) plated onto 12-well plates. Cells were serum-starved for 24 hours before experimentation. Confluent monolayers of CRL-2592 were wounded using a P200 (200-μL) pipette tip. The wounds were washed and picture-documented prior to peptide treatment. Peptides were added to a final concentration from 20 to 40 μg/ml. Cells were kept in an incubator at 37° C., 5% CO₂, and 92% humidity, except when images were being captured for a short period at room temperature. Wound closure was followed at 6-hour and 10-hour time points. PBS-treated wounds were used as negative controls for comparison purposes.

TABLE 7

Effect of peptides on human skin epithelial wound closure in vitro					
Compound	0 hr	6 hr		10 hr	
	W-size*	W-size	% closure	W-size	% closure
PBS-1	36	29	19.40%	21	41.70%
PBS-2	52	42	19.20%	30	42.30%
SEQ ID NO: 14	25	12	52%	2.75	89%
SEQ ID NO: 5	48	39	19%	30	37.50%

*W-size: wound size (arbitrary)

In vitro monolayer wound closure is a result of cell migration, which is important in many biological processes such as embryogenesis, angiogenesis, inflammatory reactions and wound repair. These processes are thought to be regulated by interactions with other cells, cytokines and ECM proteins. As shown in Table 7, SEQ ID NO:14 significantly induces wound closure compared to the effects of PBS alone. Such activity is peptide-specific as well as cell type-specific since SEQ ID NO:14 does not induce wound closure in a human skin fibroblast monolayer (data not shown). SEQ ID NO:5 is also a collagen inducer, but does not enhance wound closure or epithelial cell migration to any great extent compared to the effects of PBS alone. The fact that SEQ ID NO:14 induced cell migration or wound closure in a manner specific to skin epithelial cells (i.e. does not recruit fibroblasts) may add an advantage to using this peptide for skin care, since it is believed that the recruitment of large numbers of active fibroblasts to a wound site results in excess deposition and contraction of tissue resulting in scarring.

All of the compositions or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the methods described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention.

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35 40 45

Gly Glu Arg Gly Leu Pro Gly Leu Gln Gly Val Ile Gly Phe Pro Gly
50 55 60

Met Gln Gly Pro Glu Gly Pro Gln Gly Pro Pro Gly Gln Lys Gly Asp
65 70 75 80

Thr Gly Glu Pro Gly Leu Pro Gly Thr Lys Gly Thr Arg Gly Pro Pro
85 90 95

Gly Ala Ser Gly Tyr Pro Gly Asn Pro Gly Leu Pro Gly Ile Pro Gly
100 105 110

Gln Asp Gly Pro Pro Gly Pro Pro Gly Ile Pro Gly Cys Asn Gly Thr
115 120 125

Lys Gly Glu Arg Gly Pro Leu Gly Pro Pro Gly Leu Pro Gly Phe Ala
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Gly Asn Pro Gly Pro Pro Gly Leu Pro Gly Met Lys Gly Asp Pro Gly
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Glu Ile Leu Gly His Val Pro Gly Met Leu Leu Lys Gly Glu Arg Gly
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Phe Pro Gly Ile Pro Gly Thr Pro Gly Pro Pro Gly Leu Pro Gly Leu
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Gln Gly Pro Lys Gly Asp Lys Gly Asp Gln Gly Val Ser Gly Pro Pro
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Gly Val Pro Gly Gln Ala Gln Val Gln Glu Lys Gly Asp Phe Ala Thr
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275 280 285

Pro Gly Lys Asp Gly Asp Lys Gly Glu Lys Gly Ser Pro Gly Phe Pro
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Gly Glu Pro Gly Tyr Pro Gly Leu Ile Gly Arg Gln Gly Pro Gln Gly
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Glu Lys Gly Glu Ala Gly Pro Pro Gly Pro Pro Gly Ile Val Ile Gly
325 330 335

Thr Gly Pro Leu Gly Glu Lys Gly Glu Arg Gly Tyr Pro Gly Thr Pro
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Gln Pro Gly Pro Pro Gly Leu Pro Val Pro Gly Gln Ala Gly Ala Pro

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		835					840					845				
Gly	Leu	Pro	Gly	Ile	Thr	Gly	Gln	Ser	Gly	Leu	Pro	Gly	Leu	Pro	Gly	
	850					855					860					
Gln	Gln	Gly	Ala	Pro	Gly	Ile	Pro	Gly	Phe	Pro	Gly	Ser	Lys	Gly	Glu	
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	20	25 30
Ser His Leu Gly Gln Ser Tyr Ala Asp Arg Asp Val Trp Lys Pro Glu		
	35	40 45
Pro Cys Gln Ile Cys Val Cys Asp Ser Gly Ser Val Leu Cys Asp Asp		
	50	55 60
Ile Ile Cys Asp Asp Gln Glu Leu Asp Cys Pro Asn Pro Glu Ile Pro		
65	70	75 80
Phe Gly Glu Cys Cys Ala Val Cys Pro Gln Pro Pro Thr Ala Pro Thr		
	85	90 95
Arg Pro Pro Asn Gly Gln Gly Pro Gln Gly Pro Lys Gly Asp Pro Gly		
	100	105 110
Pro Pro Gly Ile Pro Gly Arg Asn Gly Asp Pro Gly Ile Pro Gly Gln		
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Pro Gly Ser Pro Gly Ser Pro Gly Pro Pro Gly Ile Cys Glu Ser Cys		
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Pro Thr Gly Pro Gln Asn Tyr Ser Pro Gln Tyr Asp Ser Tyr Asp Val		
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Lys Ser Gly Val Ala Val Gly Gly Leu Ala Gly Tyr Pro Gly Pro Ala		
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Gly Pro Pro Gly Pro Pro Gly Pro Pro Gly Thr Ser Gly His Pro Gly		
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Ser Pro Gly Ser Pro Gly Tyr Gln Gly Pro Pro Gly Glu Pro Gly Gln		
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Ala Gly Pro Ser Gly Pro Pro Gly Pro Pro Gly Ala Ile Gly Pro Ser		
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Gly Pro Ala Gly Lys Asp Gly Glu Ser Gly Arg Pro Gly Arg Pro Gly		
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Glu Arg Gly Leu Pro Gly Pro Pro Gly Ile Lys Gly Pro Ala Gly Ile		
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Pro Gly Phe Pro Gly Met Lys Gly His Arg Gly Phe Asp Gly Arg Asn		
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Gly Glu Lys Gly Glu Thr Gly Ala Pro Gly Leu Lys Gly Glu Asn Gly		
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Leu Pro Gly Glu Asn Gly Ala Pro Gly Pro Met Gly Pro Arg Gly Ala		

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1460						1465								

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What is claimed is:

1. A composition comprising at least one tetrapeptide capable of inducing production of extracellular matrix proteins, wherein the amino acid sequence of the tetrapeptide consists of SEQ ID NO:1 or SEQ ID NO:14, said sequence having an amidated carboxy-terminus, and a pharmaceutically acceptable carrier.

2. The composition of claim 1, wherein the tetrapeptide is SEQ ID NO:1.

3. The composition of claim 1, wherein the tetrapeptide is SEQ ID NO:14.

4. The composition of claim 1, wherein the tetrapeptide is present in an effective concentration ranging from about 0.1 µg/mL to about 50 µg/mL.

5. The composition of claim 1, wherein the composition is in the form of an aerosol, emulsion, lotion, cream, paste, ointment or foam.

6. The composition of claim 1 which further comprises a retinoid.

7. A method for stimulating the production of collagen in a human in need thereof, the method comprising administering to said human a therapeutically effective amount of a composition comprising at least one tetrapeptide capable of inducing production of extracellular matrix proteins, wherein

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the amino acid sequence of the tetrapeptide consists of SEQ ID NO:1 or SEQ ID NO:14, said sequence having an acid or amidated carboxy-terminus, and a pharmaceutically acceptable carrier.

8. The method of claim 7 wherein the composition is administered topically to a site of damaged skin.

9. The method of claim 7, wherein the therapeutically effective concentration is in the range of about 0.1 µg/mL to about 50 µg/mL of tetrapeptide.

10. The method of claim 7, wherein the administering to said human a therapeutically effective amount of the composition promotes wound healing of damaged skin.

11. The method of claim 10, wherein said damaged skin is a result of aging, wrinkling, disease, injury, trauma, or surgery.

12. A skincare composition comprising a tetrapeptide consisting of SEQ ID NO:1 or SEQ ID NO:14, said sequence having an amidated carboxy-terminus, and a dermatologically acceptable carrier.

13. The skincare composition of claim 12, wherein the composition is in the form of an aerosol, emulsion, lotion, cream, paste, ointment, or foam.

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